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Introduction

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The discovery of antibiotics in the 20th century has saved millions of lives, by boosting the fight against pathogenic microorganisms. However, the effectiveness of these compounds is significantly reduced when the pathogens develop different resistances (Ferri et al., 2015; Prestinaci et al., 2015). Some pathogens even become multidrug-resistant (MDR) and start to threaten human health, such as the MRSA (methicillin-resistant *Staphylococcus aureus*), VRE (vancomycin-resistant *Enterococcus faecalis*), MDR *Salmonella* Typhimurium phage type DT10, fluoroquinolones and tetracyclines resistant *Campylobacter* spp., *Klebsiella pneumonia* (Ferri et al., 2015), third-generation cephalosporin-resistant *Escherichia coli* and MDR *Pseudomonas aeruginosa* (Tenover, 2006). To fully control the infections caused by MDR pathogens, searching for new antimicrobials, especially those with novel inhibition mechanisms and inducing less resistance, remains essential (Bassetti and Righi, 2015; Draenert et al., 2015). Lantibiotics are a class of antimicrobial peptides, which can efficiently inhibit the growth of Gram-positive bacteria (Bierbaum and Sahl, 2009). Lantibiotics can bind to the pyrophosphate group of lipid II molecule to inhibit the cell wall synthesis and subsequently form pores in the membrane (Breukink et al., 1999; Hasper et al., 2006). Because the pyrophosphate molecule is difficult to be modified by bacteria and the dual mode of action is fatal for the bacteria, resistance to lantibiotics is rarely observed (Breukink and de Kruijff, 2006; Draper et al., 2015). Therefore, lantibiotics and variants thereof might be developed as potential antibiotics.

Lanthipeptides are (methyl)lanthionine containing peptides, which belong to the large family of ribosomally synthesized and post-translationally modified peptides (RiPPs) (Arnison et al., 2013). Lantibiotics, a subclass of lanthipeptides with high antimicrobial activity, undergo intensive

posttranslational modifications (Willey and van der Donk, 2007; Knerr and Donk, 2012). The dehydration of serines/threonines and the subsequent (methyl)lanthionine formation are their characteristic modifications. So far, about 100 different lanthipeptides have been described. Based on the different enzymes that catalyze these modifications, they are divided into four classes. And only the Class I and Class II lanthipeptides possess antimicrobial activity, while Class III and IV lanthipeptides display other biological effects. Those antimicrobial active lanthipeptides are called lantibiotics (Schnell et al., 1988). **Table 1** shows the classification, representative members and potential applications of lanthipeptides. Notably, some lanthipeptides *e.g.* MU1140 (mutacin 1140) (Ghobrial et al., 2010), NAI-107 (microbisporicin) (Jabés et al., 2011; Lepak et al., 2015), NVB302 (Crowther et al., 2013) and duramycin (Jones and Helm, 2009; Elvas et al., 2015) are already in late pre-clinical or clinical trials and might be applied as medicines soon.

Table 1. Classification of lanthipeptides. Adapted from Dischinger et al., 2014.

Class/Characters/ Names	**Producers**	**Potential applications**
Class I Lanthipeptides (LanB and LanC modified)		
Nisin A/Z	*Lactococcus lactis* ATCC 11454/ *Lactococcus lactis* N8	Described below
Epidermin	*Staphylococcus epidermidis*	Prevent adhesion of coagulase-negative staphylococci to catheters
Gallidermin	*Staphylociccus gallinarum* (F16/P57) TÜ3928	Prevent acne; prophylactic against implantate associated infections
Mutacin 1140	*Streptococcus mutans*	In preclinical development for the treatment of Gram-positive infections
Pep5	*Staphylococcus epidermidis* 5	Prevent adhesion of coagulase-negative staphylococci to catheters
Class II Lantibiotics (LanM modified)		
Lacticin 481	*Lactococcus lactis* CNRZ 481	Not described

Nukacin ISK-1	*Staphylococcus warneri*	Not described
Salivaricin	*Streptococcus salivarius* 20P3	The producing strain *S. salivarius* was added to milk drink or chewing gums for oral care
Mersacidin	*Bacillus amyloliquefaciens* HIL Y-85	Eradicate the nasal colonization by MRSA
Cinnamycin	*Streptomyces cinnamoneus*	Active against retroviruses
Duramycin	*Streptomyces cinnamoneus*	Treatment of cystic fibrosis by inhalation; anti-inflammatory
Lacticin 3147	*Lactococcus lactis subspec. lactis* DPC 3147	Prevent acne; anti-caries
Haloduracin	*Bacillus halodurans* C-125	Not described
Lichenicidin	*Bacillus licheniformis* DSM13/ATCC14580/VK21	Not described
Prochlorosins	*Prochlorococcus* MIT9313	Not described
Class III Lanthipeptides (LabKC modified)		
SapB	*Streptomyces coelicolor*	Biosurfactants
SapT	*Streptomyces tendae*	Biosurfactants
Labyrinthopeptin	*Actinomadura namibiensis* DSM 6313	Numb neuropathic pain in a mouse model; anti-dengue-virus
Class IV Lanthipeptides (LanL modified)		
Venezuelin	*Streptomyces venezuelae*	Not described

Nisin is the first described- (Rogers, 1928) and also a commercially used lantibiotic. As nisin can efficiently inhibit the growth of food-spoilage pathogens, *e.g. Listeria* species and *Clostridium botulinum*, it has been wildly used as food preservative in dairy products, seafood products, vegetable foods and alcoholic beverages (Gharsallaoui et al., 2015; Bali et al., 2014). Nisin is also commercially used in animal care to prevent mastitis (Wipe Out | Immucell) by killing the causative pathogens *Staphylococcus aureus* and *Streptococcus agalactiae*. Nisin could also be applied to treat human diseases. For instance, nisin is a potential candidate to be applied in oral medicine. Combination of nisin with fluoride or free D-amino acids could kill *Staphylococcus mutans* and

affect the biofilm formation more efficiently, and might become an effective strategy against caries (Tong et al., 2014). Combining nisin with MTAD (a common intracanal irrigant) or common antibiotics could be used in root canal therapy, especially in treating the infections caused by *Enterococcus faecalis* (Tong et al., 2014). Nisin might also be used as a cancer therapeutic for treating head and neck squamous cell carcinoma (HNSCC) (Joo et al., 2012), because nisin could potentially reduce HNSCC tumorigenesis, by inducing preferential apoptosis, decreased cell proliferation and cell cycle arrest, partly aided by a proapoptotic cation transport regulator (CHAC1). A high content nisin (nisin ZP) showed better anti-HNSCC effects, and long periods of treatment could extend survival of mice, while no apparent side effects were found (Kamarajan et al., 2015). As nisin is very stable and active in an acidic environment, it could be a good option in treatment of peptic ulcers (Delves-Broughton et al., 1996).

Nisin is produced by *Lactococcus lactis*. The structural, modification, regulation and immunity genes of nisin are located on one transposon, *Tn5276*, and organized in four operons, *nisABTCIPRK*, *nisI*, *nisRK* and *nisFEG*. *nisA* encodes prenisin; *nisBTCP* encode the modification and maturation machinery; *nisRK* encode the two component regulatory system while *nisI* and *nisFEG* encode proteins related to immunity of the host to nisin (for review see (Lubelski et al., 2008b)). **Figure 1** shows the maturation process of nisin. After ribosomal synthesis, prenisin is first modified by NisB. NisB exists as a dimer (Mavaro et al., 2011) and each monomer contains an N-terminal glutamylation region and a C-terminal glutamate-elimination region (Ortega et al., 2015). The N-terminal region tightly binds the FNLDL box in the leader part of prenisin, and catalyzes a glutamyl-tRNA involved glutamination of serines and threonines in the core peptide part. Subsequently, the glutamate is removed by the C-terminal domain of NisB and serines and threonines are dehydrated to dehydroalanines (Dha) and dehydrobutyrines (Dhb) (Garg et al., 2013; Ortega et al., 2015). The dehydrated residues are coupled to cysteines by a sulfhydryl addition catalyzed by NisC and a zinc ion, and the (methyl)lanthionine rings are formed (Kuipers et al., 1993a; Li et al., 2006). NisB and NisC work in an

alternating fashion and the modification of prenisin is performed in an N- to C-terminus direction (Lubelski et al., 2009). The modified peptide is transported outside the cell by NisT (Qiao and Saris, 1996), and NisP is a proteinase, which dedicatedly cuts off the leader part and activates nisin (van der Meer et al., 1993). The expression of *nisA*, *nisBTCP* and *nisFEG* genes is controlled by the NisRK regulatory system (Kuipers et al., 1995a; de Ruyter et al., 1996a). NisK is a histidine sensor kinase (Engelke et al., 1994) localized in the cytoplasmic membrane, and when NisK senses nisin in the medium, the histidine is auto-phosphorylated (Kuipers et al., 1995a). Subsequently, the phosphate is transferred to NisR, which is a response regulator (van der Meer et al., 1993) and activates the transcription of *nisA*, *nisBTCP* and *nisFEG*.

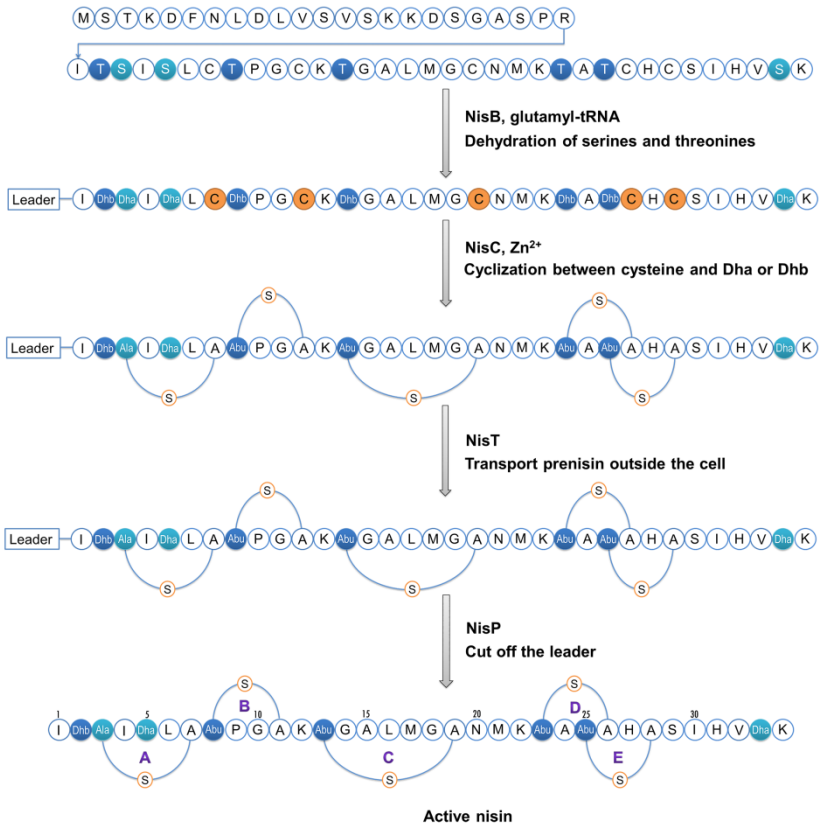

Figure 1. Posttranslational modification and maturation process of nisin.

The bioengineering of lantibiotics has been conducted intensively (Kuipers et al., 1992, 1995b, 1996; Lubelski et al., 2008b; Ross and Vederas, 2011; Tabor, 2014), by *in vivo* engineering, chemical synthesis or *in vitro* biosynthesis. *In vitro* biosynthesis includes *in vitro* mutasynthesis, which means that the mutated peptide substrates are modified by related synthetases (*e.g.* LacM, HalM1 and HalM2) *in vitro*, and semi- synthetic, which means lantibiotics or parts thereof were hybridized with other compounds *in vitro* by click chemistry (*e.g.* nisin (1-12)-vancomicin hybrid, NVB302) (Escano and Smith, 2015). The engineering of nisin is mainly conducted by *in vivo* engineering and a large number of variants have been created (Lubelski et al., 2008b; Molloy et al., 2012). **Table 2** shows part of the mutants of nisin described in the literature. Nisin A and nisin Z have one amino acid difference in position 27 (nisin A has His, while nisin Z has Asn), but showed exactly the same activity in liquid culture (Rollema et al., 1995), which indicates that the sequence of nisin is modifiable. The first reported mutations were made in nisin Z in 1992 (Kuipers et al., 1992). The mutant Dha5Dhb showed reduced antimicrobial activity, but it was later indicated to possess higher stability against acid (Rollema et al., 1995). The double mutants M17Q/G18T (Dhb) showed enhanced activity against particular indicators and the inhibition spectrum was different depending on the dehydration of T18 (Kuipers et al., 1992). The mutants N27K and H31K of nisin Z displayed similar activity to wild type, but the solubility of N27K was increased 4 times, while that of H31K increased 7 times (Rollema et al., 1995). Three kinds of tryptophan mutants (I1W, M17W and V32W) were made in nisin Z, which increased the fluorescence of nisin. The mutants showed 2-3 times reduced activity against *Micrococcus flavus* and 3-8 times reduced activity against *Streptococcus thermophilus* (Kuipers et al., 1995b; Breukink et al., 1998). However, there are several mutants with improved antimicrobial activities. The mutant T2S showed 2 times enhanced activity than nisin Z against *M. flavus* and *S. thermophilus* (Wiedemann et al., 2001). Two triple

mutants in Ring A of nisin A (KSI and KFI) resulted in increased activity against particular bacteria (Rink et al., 2007b). The K12 between ring B and ring C of nisin was mutated to alanine by Molloy *et al.* and the mutant showed two or four times increased activity against several different bacteria, including an MRSA strain (Molloy et al., 2013).

Table 2 Activities and properties of descried mutants of nisin (excluding the hinge region mutants). Red denotes the fold of increased activity or improved characters; blue denotes fold of decreased activity; ND, not described.

Nisin variants	**Nisin Z (Asn27)**										**Nisin A (His 27)**		
Wild type	Dha5	M17/G18	M17/G18	N27	H31	I1	M17	V32	T2	I/Dha/L	I/Dha/L	K12
Mutants	Dhb	Q/T	Q/Dhb	K	K	W	W	W	S	K/Dha/I	KFI	A
M. flavus	2	2	1	1	1.5	1.6	2	2.7	2	ND	ND	ND
S. thermophilus	10	4	1	1	1.5	7	8	3.3	2	ND	ND	ND
B. cereus	5	4	1	ND	ND	ND	ND	ND	ND	ND	ND	4
L. lactis	9	ND	ND	1	2	ND	ND	ND	ND	1.5	ND	2/4
Lactobacillus johnsonii	ND	ND	ND	ND	ND	ND	ND	ND	ND	2	10	ND
Leuconostoc mesenteroides	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	9	ND
Enterococcus faecalis	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2
Streptococcus pyogenes	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2
S. aureus MRSA ST 534	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2
Other properties	**Stability**	ND	ND	**Solubility**		**Fluorescence**			ND	ND	ND	ND

The hinge region (NMK) is the most flexible part in nisin. The double mutant PPK showed 3 times reduced inhibitory activity against *M. flavus* and 25 times reduced activity against *S. thermophiles*, and the pore formation activity on liposomes was abolished (Wiedemann et al., 2001; Hasper et al., 2004), which means that the flexibility of hinge region is important for the pore formation activity of nisin. By introducing one more positively charged residues into the hinge region (N20K, M21K) of nisin Z, the variants showed slightly reduced activity against *M. flavus* and *S. thermophilus,* but started to be active against particular Gram-negatives. The mutants N20E, N20V, M21E and N20A/M21K/Dhb/K22G showed dramatically reduced activity against these two indicators. The solubility of N20K and M21K was higher than that of nisin Z at a wide pH range (pH 2-pH 11), while the solubility of N20H and M21H was lower than that of nisin Z above pH 5. The mutants N20Q and M21G were found to display increased stability at pH 6-9 compared to nisin Z (Yuan et al., 2004). Two positive hinge region variants (K22T and M21V) were created by intensive mutagenesis. The K22T mutant showed 2 times increased activity against several indicators, *e.g.* the mastitic pathogen *Streptococcus agalactiae*, *Clostridium difficile* ribotype 027, MRSA, heterogenous vancomycin-intermediate *S. aureus* (hVISA) and *L. lactis* spp cremoris HP, but not *L. monocytogenes*, VRE and *B. cereus*. Its activity is different, when testing against different strains of the same species. Notably, the M21V mutant displayed 2 times increased activity against all the tested strains, and might be applied in both food and medical industry (Field et al., 2010). The hinge region variants SVA and NAK did not show significant improved activity in liquid culture but were found to perform faster diffusion in complex polymers, and they were suggested to be used to control *L. monocytogenes* in polysaccharides containing food (Rouse et al., 2012). After intensive site-saturation mutagenesis, it was indicated that small and chiral amino acids are optimal constitution of the hinge region. And the mutants AAA and SAA displayed enhanced bioactivity

against particular indicators (Healy et al., 2013).

Lantibiotics have been overexpressed by different systems. The overproduction systems of nisin are constructed in both *L. lactis* and *E. coli*. The first expression system is constructed by Kuipers *et al.* in *L. lactis*, and is controlled by PlacZ promoter (Kuipers et al., 1992). A widely used nisin variants production system was first described by the group of Moll *et al.*. The modification and transportation enzymes (NisBTC) of nisin were encoded in the plasmid pIL3BTC (Kluskens et al., 2005), separated from the structure gene containing plasmid (pNZnisA derivatives) (Rink et al., 2005), and the expression of the enzymes and peptides are controlled by Pnis promoter in *L. lactis* NZ9000 harboring NisR and NisK (Kuipers et al., 1997). NisBTC have broad substrate specificity, which facilitate the expression and modification of nisin variants and other lanthionine containing peptides (Kluskens et al., 2005; Kuipers et al., 2004; Rink et al., 2007a; Majchrzykiewicz et al., 2010b). Furthermore, the system was extended to include additional enzymes (*e.g.* GdmD, LtnJ), to introduce extra modifications (*e.g.* C-terminal aminovinyl-cysteine, D-alanine) into the lantibiotics (van Heel et al., 2013). **Figure 2** shows the extended lantibiotic production system in *L. lactis*. Nisin was also successfully produced in *E. coli* with high production levels (Garg et al., 2013; Shi et al., 2011). With the same system, four different variants of prochlorosins and the two-component lantibiotic haloduracin were also overexpressed (Shi et al., 2011). Another two-component lantibiotic, lichenicidin, was successfully expressed in *E. coli* as well (Caetano et al., 2011; Kuthning et al., 2015). Beside these heterologous expression systems, some lantibiotics are overexpressed in their host or host related strains. For example, an expression and random mutagenesis system for lacticin 3147 was constructed in *L. lactis* (Field et al., 2007). The extracellular pregallidermin protease GdmP deletion strain, *Staphylococcus gallinarum* ΔP, showed high

production of pregallidermin (1.95 g/l) after medium optimization (Medaglia and Panke, 2010). Mersacidin was produced in *Bacillus amyloliquefaciens* FZB42, which is a closely related strain with the producer of mersacidin (Herzner et al., 2011).

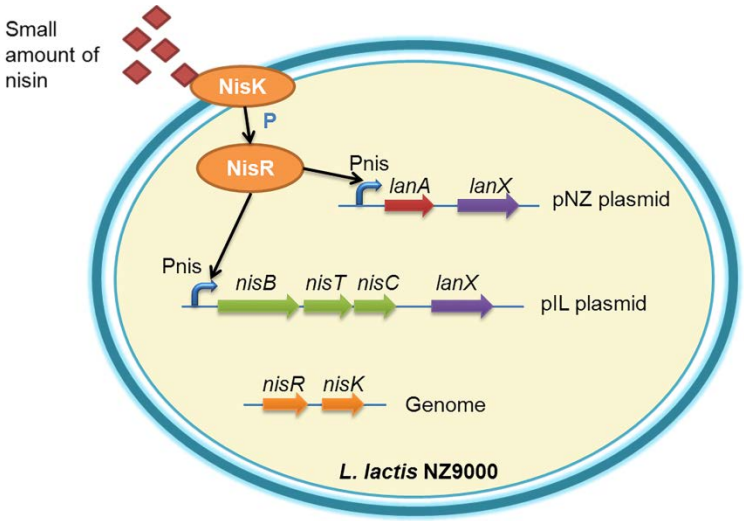

Figure 2. Lantibiotics production system in *L. lactis*. P: phosphate; Pnis: nisin promoter; *lanX*: other modification enzyme, which could be introduced to this system; *lanA*: target peptides guided by leader of nisin. **(van Heel et al., 2013; Kluskens et al., 2005)**.

Incorporation of non-canonical amino acids (ncAAs) into proteins provides a novel protein engineering method. It could be conducted by two kinds of *in vivo* methods or an *in vitro* method. NcAAs could be assigned into a protein at particular positions, where the stop codon(s) or artificially programmed quadruplet codon(s) exist, which is called "genetic code expansion" (for review (Hoesl and Budisa, 2011, 2012; Terasaka et al., 2015)). Recently, the arginine codon (AGG) was redefined in *E. coli*, and arginine analogs were efficiently incorporated into proteins in response to AGG, aided by an engineered pyrrolysyl-tRNA synthetase (PylRS) and the AGG-reading $tRNA^{Pyl}_{CCU}$

molecule (Mukai et al., 2015). The “genetic code expansion” method has been used in the *E. coli* expression system for lantibiotics. Phe26 of prochlorosin A3.2 was changed to p-benzoyl-L-Phe, by introducing an amber stop codon (TAG) and an orthogonal tRNA/tRNA synthetase pair (Shi et al., 2011). Another *in vivo* incorporation method is constructing an auxotrophic strain for particular amino acids. During translation, the analogues can be incorporated instead of normal amino acids (Budisa, 2013). The analogues of Met, Pro, and Trp have been successfully incorporated into lichenicidin Bliα and Bliβ, with three different auxotrophic strains of *E.coli* (Oldach et al., 2012). Non-canonical amino acids could also be incorporated by *in vitro* mutasynthesis. Lacticin 481 analogues containing ncAAs were generated in this way. The LctA analogues were first chemically synthesized and then modified by LctM *in vitro*. Two of the mutants Trp19Nal (naphthylalanine) and Phe23hPhe (homophenylalanine) showed enhanced activity against *L. lactis* HP (Levengood et al., 2009). All these modification methods have the potential of being used for extensive lantibiotic engineering yielding a wide variety of structural and functional variants that could generate new desirable antibacterial activities to fight pathogens.

Outline of the thesis:

Chapter 1 is the general introduction concerning lantibiotics, nisin, the biosynthesis of nisin, current development in the engineering of nisin, the expression systems for lantibiotics and non-canonical amino acid incorporation.

Chapter 2 describes the purification of the complex constituted by the dehydratase (NisB) and its substrate prenisin from *L. lactis*, characterization of the complex with Western blot, the stability of the complex and the feasibility of using cryo-EM to analyze the structure of the complex.

In **Chapter 3**, the hinge region of the lantibiotic nisin was engineered by varying the length of it, from one to six amino acids. The antimicrobial activities of the variants were tested against 10 different indicator strains, either in liquid medium or using agar plates. The importance and effects of the length of hinge region on the antimicrobial activity of nisin were indicated and possible applications of the new variants were discussed.

Chapter 4: To enhance the activity of lantibiotics against Gram-negative bacteria we made fusions of nisin with peptides that have antimicrobial activity against Gram-negative strains, and antimicrobial activities against both Gram-negative and Gram-positive bacteria were tested. One of the hybrids was found to possess increased activity against Gram-negative bacteria. The advantage of this method and the potential of creating more improved fusions were indicated.

Chapter 5: Three different tryptophan analogues were incorporated into nisin. To achieve this we combined a tryptophan analogue incorporation system with the nisin modification system in an auxotrophic *L. lactis* strain. The incorporation efficiency was analyzed. The biochemical properties and antimicrobial activities of the variants were compared to address the effects of the analogues.

In **Chapter 6**, the potential to develop lantibiotics as new antimicrobial agents is discussed; moreover, the studies in chapters 2-5 are summarized, and further interpreted and the future developments of lantibiotic engineering are discussed.